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Small, Antibacterial and Large, Inactive Peptides of Neutrophil Granules Share Immunoreactivity to a Monoclonal Antibody

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Monoclonal antibodies were raised against a bactericidal protein fraction that was purified from an extract of bovine neutrophil granules and that was previously shown (A. Savoini, R. Marzari, L. Dolzani, D. Serranò, G. Graziosi, R. Gennaro, and D. Romeo, Antimicrob. Agents Chemother. 26:405–407, 1984) to inhibit bacterial DNA synthesis. One of these antibodies, BP97, was covalently linked to Affi-Gel 10 and was used for immunoaffinity chromatography of granule extracts. Elution of the bound proteins, followed by reversed-phase high-performance liquid chromatography, generated several peptides whose molecular weights fell in the range of 1,600 to 64,000 and which reacted to BP97 but not to other mouse monoclonal or polyclonal antibodies. The reaction to BP97 appeared to be specific, inasmuch as a full panel of cationic oligo- or polypeptides was not recognized by this monoclonal antibody. Among the purified granule polypeptides, the more cationic ones (with molecular weights of 4,300 to 8,000) inhibited the growth of *Escherichia coli* at a MIC of 12 to 50 μg/ml. In addition, a 1,600-molecular-weight, highly cationic peptide also inhibited the growth of *Staphylococcus aureus* and *Staphylococcus epidermidis* at MICs of 3 to 8 μg/ml.

Neutrophilic leukocytes are one of the major cellular defense mechanisms that protect animals from microbial infections (1, 13). Invading bacteria are sequestered by neutrophils into phagocytic vacuoles, where they may be killed and eventually destroyed. This process is accomplished through the release into the vacuoles of toxic substances which are likely to include cationic polypeptides and which exhibit significant in vitro bactericidal activity (5, 6, 8, 18–20, 21, 23, 25). These peptides are stored in neutrophil granules, from which they may be discharged into the phagocytic vacuoles on fusion of the granules with the vacuoles.

In bovine neutrophils powerful bactericidal polypeptides are stored in a special class of storage granules (7). These polypeptides have been extracted from the granules and resolved into subfractions with various levels of effectiveness against gram-positive and gram-negative bacteria (8, 18). A fraction with two major components (fraction I) has been shown to arrest the growth of a variety of microorganisms, including clinical isolates of *Staphylococcus* spp. and *Enterobacteria* spp. that are resistant to one or more of the commonly used commercial antibiotics (18). The mechanism of action of this fraction involves inhibition of bacterial DNA synthesis (18).

We prepared monoclonal antibodies (MAbs) against fraction I, and one of these antibodies (MAb BP97) was used for immunoaffinity chromatography of granule extracts. By this method we resolved several polypeptides from the extracts that shared immunoreactivity to BP97 but that possessed different molecular weights, a positive charge density, and antimicrobial activity.

The availability of MAb BP97, as well as those of the other MAbs that are produced, may be important for investigations of the relationships among the various antimicrobial peptides of bovine neutrophil granules, their possible evolution from a common precursor molecule(s) by proteolytic cleavage, and their distribution in neutrophils and other phagocytes of various animal species.

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MATERIALS AND METHODS

Extraction of neutrophil granules. Neutrophils were purified from freshly collected bovine blood, and granules were isolated from postnuclear supernatants as reported previously (7). Granule pellets were suspended by homogenization in 0.2 M acetate–5 mM EDTA (pH 4; 0.3 to 0.7 ml per granule derived from 10^9 neutrophils), subjected to a brief sonication, and stirred for 2 h in an ice bath. The insoluble residue was sedimented for 30 min at 30,000 × *g* and reextracted overnight with 50 to 100 ml of buffer as described above, and the solubilized proteins were combined with the first extract. This material, which is referred to as the acetate-EDTA granule extract, was dialyzed against large volumes of distilled water acidified with a few drops of concentrated HCl (ca. pH 3) in membrane tubing (nominal molecular weight cutoff, 1,000; Spectrapore 6; Spectrum Medical Industries, Los Angeles, Calif.). The dialyzed extract was then vacuum dried, suspended in 100 to 200 ml of 10 mM Na_2HPO_4–0.2 M NaCl (pH 7), and clarified by centrifugation for 40 min at 30,000 × *g*.

On one occasion, extraction of the granule pellets was carried out in the presence of protease inhibitors, which were selected for their specificity against acid granule proteases, mainly cathepsins B and D (2), because they potentially act at the pH of the extraction medium. Two procedures were adopted. In the first one, the acetate-EDTA extraction buffer was supplemented with 10 μg each of leupeptin and pepstatin A (both from Sigma Chemical Co., St. Louis, Mo.) per ml. The extract was then dialyzed against HCl-acidified distilled water containing 5 mM EDTA and 1 μg each of leupeptin and pepstatin A per ml and lyophilized. Alternatively, the granules were extracted at a lower pH (ca. 2) with 0.1% trifluoroacetic acid (TFA), which is a good solvent for the granule peptides (see below), that

was supplemented with 10 μg each of leupeptin and pepstatin A per ml and immediately lyophilized.

Carboxymethyl cellulose chromatography. A portion of the acetate-EDTA granule extract was applied to a column (1.5 by 20 cm) of carboxymethyl cellulose (CM 52; Whatman Inc., Maidstone, Kent, United Kingdom) equilibrated in 10 mM Na_2HPO_4–0.2 M NaCl (pH 7). After the column was washed extensively with the same buffer, elution of the bound proteins was first carried out with 0.3 M NaCl and then with 1 M NaCl (in Na_2HPO_4 [pH 7]). The fractions that were obtained during the two elution steps were combined into pools 2 and 3, respectively. These pools have previously been shown to collect all the cationic bactericidal proteins extracted from the granules of bovine neutrophils (8).

Production of MAbs. Two female BALB/c mice were each injected intraperitoneally with 1 mg of fraction I (18) emulsified in Freund complete adjuvant. This was followed by two booster shots of 0.5 mg of fraction I, one in Freund incomplete adjuvant and the other in 10 mM sodium phosphate–0.15 M NaCl (phosphate-buffered saline; pH 7.2), administered at 14-day intervals. Spleens were removed 4 days after the final injection. Spleen cells were fused with X63Ag8.6.5.3 myeloma cells (12), and the resulting hybridoma cells were cloned into 96-cell dishes (Costar, Cambridge, Mass.) over a feeder layer of mouse spleen cells in the presence of hypoxanthine-aminopterin-thymidine selective medium. Culture supernatants were screened for anti-fraction I antibodies by the dot blot method (see below).

MAb BP97 was prepared from the spent media of expanded cultures (500 ml) that were grown to the stationary phase. After cell sedimentation, the immunoglobulins were concentrated by precipitation with 50% saturated ammonium sulfate (pH 7) and further purified by affinity chromatography through Affi-Gel protein A (MAPS II kit; Bio-Rad Laboratories, Richmond, Calif.).

The subclass of BP97 was determined by double immunodiffusion with subclass-specific sheep anti-mouse antibodies (Miles Scientific, Div. Miles Laboratories, Inc., Naperville, Ill.).

Immunoaffinity chromatography. After dialysis against 0.1 M sodium bicarbonate (pH 8.5), MAb BP97 (10 mg) was coupled to 4 ml of Affi-Gel 10 (Bio-Rad). Coupling of the Mab to Affi-Gel had an efficiency of over 90%. Untreated sites were blocked by mixing the gel with an equal volume of 0.1 M ethanolamine hydrochloride (pH 8). The gel was then transferred to a column, rinsed with phosphate-buffered saline containing 0.02% (wt/vol) sodium azide, and stored at 4°C.

To carry out the immunoaffinity chromatography, 10 ml of the acetate-EDTA extract (ca. 10 mg/ml) was applied to the column (0.7 by 10 cm) at a flow rate of 4 ml/h. The column was extensively washed with phosphate-buffered saline and then with 20 volumes of 10 mM sodium phosphate–0.5 M NaCl–0.2% Tween 20 (pH 7.2). All these operations were carried out at 4°C. The bound polypeptides were eluted with 0.1% TFA, and the peak fractions were immediately frozen and kept at −20°C. The solution of unbound proteins was again passed through the regenerated immunoaffinity gel until depletion of components that showed antibacterial activity, that reacted with MAb BP97, or both. This step was thus repeated 6 times, and all the fractions of bound peptides were pooled and vacuum dried.

Reversed-phase chromatography. After redissolution in 0.1% TFA, the peptides retained by the immunoaffinity column were resolved by reversed-phase chromatography on a Pep-RPC HR 5/5 column of a fast protein liquid chromatography system (Pharmacia LKB Biotechnology, Inc., Uppsala, Sweden). Elution of the peptides, which was monitored at 214 nm, was performed with a 0 to 100% gradient of acetonitrile in 0.1% TFA at a flow rate of 0.7 ml/min. The fractions of the resolved peaks were pooled and, after evaporation of the acetonitrile by bubbling oxygen-free nitrogen into the solution, were lyophilized.

Electrophoresis. Sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) (15) was carried out with either 15 or 10 to 30% linear gradient acrylamide gel slabs (length, 16.5 cm) under reducing conditions. Acid urea-PAGE (22) was conducted on 25% acrylamide gel slabs. Gels were stained with Coomassie brilliant blue R.

Western blot and dot immunobinding analyses. Proteins resolved by SDS-PAGE (15% acrylamide) were transferred electrophoretically to nitrocellulose sheets at 60 to 100 V, essentially following the conditions described by Towbin et al. (24), but the concentration of methanol in the electrophoresis buffer was reduced to 5% (23) and 0.1% SDS was added. These modifications improved the transfer, although the nitrocellulose sheet did not bind quantitatively some of the low-molecular-weight peptides. The unoccupied protein-binding sites on the sheets were blocked with a protein extract (10 mg/ml) of *Drosophila* embryos (the commonly used blocking solutions of proteins of bovine serum were not used to avoid the possibility of a cross-reaction with neutrophil peptides). The sheets were washed 5 times by soaking them in 50 mM Tris hydrochloride–200 mM NaCl (TBS; pH 7.4) containing 0.1% Tween 20 and then were incubated for 1 h at room temperature with MAb BP97 under continuous rocking, followed by 5 washings with the same buffer (Tween 20 was present only in the first washing). The MAb complexes were then visualized by incubation with biotinylated sheep anti-mouse immunoglobulins, streptavidin-biotinylated peroxidase complex and H_2O_2, and 4-chloro-1-naphthol as substrates (Amersham International, Little Chalfont, United Kingdom). Controls were run by omitting the reaction with BP97 prior to treatment with secondary antibody or by replacing BP97 with a mouse MAb (immunoglobulin G1 [IgG1] subclass) against the wheat germ protein gliadin or with the total IgG fraction of normal mouse serum.

Dot immunobinding analyses were performed by a standard technique (10). Briefly, 1 μl of antigen solution (from 1 to 100 ng) was delivered onto a nitrocellulose disk placed on the bottom of a flat well of a microtiter plate. Blocking of the unreacted sites, washing, and incubation with MAb BP97 were carried out as described above for the trans-blotted sheets. The MAb complexes were visualized with biotinylated sheep anti-mouse antibodies and streptavidin–biotin–β-D-galactosidase in the presence of 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside as the substrate (Amersham). A series of controls was carried out by (i) omitting the treatment with BP97 before the addition of sheep antibody; (ii) replacing BP97 with a mouse MAb against gliadin or with the total IgG fraction of normal mouse serum; and (iii) testing BP97 against a panel of cationic oligo- or polypeptides (from 10 ng to 1 μg), including histone (type III S), salmon protamine, RNase B (all from Sigma), polymixin (Calbiochem-Behring, La Jolla, Calif.), polylysine (Biokrom KG, West Berlin, Federal Republic of Germany), and an arginine-rich, protaminelike protein (EM1) from *Ensis minor* sperm (9).

Bacteria and evaluation of MIC. *Staphylococcus aureus* ATCC 25923, *Staphylococcus epidermidis* ATCC 12228, and *Escherichia coli* ATCC 25922 were grown in Iso-Sensitest

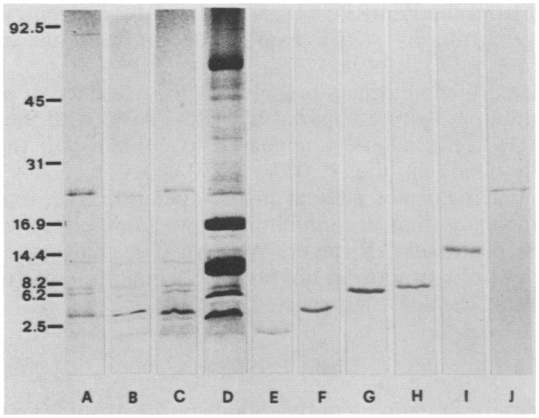

FIG. 1. SDS-PAGE of granule peptides. Coomassie brilliant blue-stained protein bands which were electrophoretically separated from a total extract of neutrophil granules (lane D) or from three different eluates from the immunoaffinity column (MAb BP97 attached to Affi-Gel 10) (lanes A to C) are shown. Lanes E to J show the migration of some components of the protein pool eluted from the immunoaffinity column and then resolved by reversed-phase high-performance liquid chromatography. The properties of these peptides, whose apparent molecular weights were 1,600, 4,300, 7,300, 8,000, 15,000, and 25,000, are described in Table 1. The numbers on the left margin are molecular weights (in thousands) of the proteins used for gel calibration.

broth (Oxoid Ltd., Basingstoke, Hampshire, United Kingdom).

MICs of granule peptides were assayed by the microdilution susceptibility test. Serial 1:1 dilutions of the peptides in 40 μl of Iso-Sensitest medium were pipetted into the wells of sterile microtiter plates, and then 10 μl of bacteria (5×10^4 CFU) in the same medium was added. Initial protein concentrations were 100 μg/ml. The plates were then incubated at 37°C for 15 to 18 h. For each peptide, the MIC was considered to be that concentration at which the microorganism tested did not exhibit visible growth.

Protein determination. Protein determination was carried out by the method of Waddel, as modified by Romeo et al. (17), with bovine serum albumin used as the standard.

RESULTS

Spleen cells of the mice immunized with fraction I containing cationic bactericidal proteins of bovine neutrophil granules (18) generated about 500 hybridoma cells. These were visible as macroscopic colonies in microwells. A total of 41 of the supernatants contained anti-fraction I antibodies, as assessed by dot immunobinding. Clone BP97 produced the most reactive supernatant, which contained a MAb of the IgG1 subclass. After subcloning, it was thus selected for expansion.

Chromatography of the acetate-EDTA granule extract through MAb BP97 covalently linked to Affi-Gel 10 caused retention of about 1 mg of protein per 100 mg of extract protein. The retained material consisted of several polypeptides with approximate molecular weights ranging from 25,000 to 1,600. The ratio between the amounts of individual proteins eluted from the affinity column, as assessed by SDS-PAGE and Coomassie brilliant blue staining of the gels, varied from experiment to experiment. This is shown in Fig. 1 (lanes A to C) for three cases.

TABLE 1. Physicochemical and bactericidal properties of some granule polypeptides reacting to MAb BP97[a]

Acetonitrile (%)	Mol wt (10^3)	Immunoreactivity	MIC (μg/ml)		
			S. aureus	*S. epidermidis*	*E. coli*
24.1 ± 0.1	1.6	+	8	3	25
24.6 ± 0.1	7.3	+	>100	>100	12
25.3 ± 0.1	8.0	±	>100	>100	50
30.5 ± 0.1	4.3	+++	>100	>100	25
33.5 ± 0.2	15	+++	>100	>100	>100
36.8	25	+++++	>100	>100	>100

[a] Peptides of the granule extract eluted from the immunoaffinity column (MAb BP97 attached to Affi-Gel 10) were resolved by reversed-phase high-performance liquid chromatography with increasing concentrations of acetonitrile in 0.1% TFA. Values of percent acetonitrile required to elute the peak fractions are means ± standard errors ($n = 2$ to 9). Molecular weights were determined by SDS-PAGE. The immunoreactivity, the extent of which is indicated by the symbols, was assayed by a dot blot method with 1 to 100 ng of antigen and 0.5 μg of MAb BP97 in 100 μl of TBS. MICs were determined with 5×10^4 bacteria per 50 μl.

When the immunoaffinity chromatography step was carried out with the granule protein fraction eluted from carboxymethyl cellulose by 1 M NaCl (pool 3) (7), a similar pattern of bound proteins was observed on SDS-PAGE, with the exception that bands with M_rs ≥15,000 were not detectable on stained gels. This suggests that at least the peptides with molecular weights in the range of 1,600 to 8,000 that are recognized by BP97 are highly cationic. These peptides actually possessed a high cathodic mobility on acid urea-PAGE (data not shown) and were eluted from a hydrophobic chromatography column ahead of the higher-molecular-weight polypeptides (Table 1).

Several lines of evidence indicated that the polypeptides that bound to the immunoaffinity column reacted specifically with the immobilized MAb. First, the column was washed extensively with buffer at a high ionic strength that contained a detergent before elution of the bound proteins with TFA. Second, on resolution by SDS-PAGE the bound proteins could be transferred electrophoretically to nitrocellulose sheets, where they reacted with BP97, but not with a MAb to wheat germ gliadin of the same subclass or with the total IgG fraction of normal mouse serum (data not shown). Immunoblotting of transferred proteins also revealed a 64,000-molecular-weight protein which likely was present at a concentration that was too low to be visible on Coomassie brilliant blue-stained gels.

Reversed-phase high-performance liquid chromatography of the proteins that eluted from the immunoaffinity column permitted the resolution of several distinct peaks. The major ones, which appeared to be homogeneous on both SDS-PAGE (Fig. 1, lanes E to J) and acid urea-PAGE (data not shown), were collected, lyophilized, and used for further studies.

In dot immunobinding assays, all the purified peptides reacted to BP97 (Fig. 2), which conversely failed to bind to any of the other cationic peptides that were used as control antigens (see above). The specificity of immunobinding of the purified peptides to BP97 was also documented by the absence of reactions with the secondary antibody alone, with a mouse MAb to the slightly cationic protein gliadin, or with the total IgG fraction of normal mouse serum.

When the peptides that reacted with BP97 by dot immunobinding (molecular weight range of 1,600 to 25,000) were tested for antibacterial activity, only the peptides that eluted from the Pep-RPC HR 5/5 column at acetonitrile concentrations lower than 30.5% exhibited MICs of 12 to 50 μg/ml

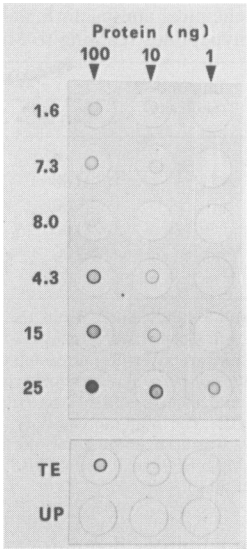

FIG. 2. Dot blot analysis of granule peptides purified by immunoaffinity and reversed-phase chromatographies, with MAb monoclonal BP97 used as a probe. The indicated amounts of peptide in 1 μg were applied to nitrocellulose disks and, after masking of unreacted sites on the nitrocellulose, were incubated with MAb BP97. The MAb complexes were visualized with biotinylated sheep anti-mouse antibodies and streptavidin–biotin–β-galactosidase in the presence of 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside as the substrate. Numbers on the left margin are apparent molecular weights (in thousands) Abbreviations: TE, total extract loaded onto the immunoaffinity column; UP, unbound proteins remaining in the extract after 6 passages through the immunoaffinity column (see text).

against *E. coli* (Table 1). In general, these peptides did not inhibit the growth of *S. aureus* or *S. epidermidis*. *S. epidermidis* is more sensitive than the *S. aureus* to the action of antibacterial peptides of bovine neutrophil granules (fraction I) (18), even at concentrations of 100 μg/ml. However, the peptide with a molecular weight of 1,600 was active against both *S. aureus* and *S. epidermidis*, with MICs of 8 and 3 μg/ml, respectively.

The close relationship between positive charge density-hydrophilicity and antibacterial activity was confirmed by comparing some minor components of the eluate from the immunoaffinity column. On SDS-PAGE these minor components migrated with identical molecular weights (4,300) (Table 2). In fact, only the more hydrophilic peptides that eluted from the Pep-RPC HR 5/5 column by concentrations of acetonitrile of <30% exerted an antibacterial action against *E. coli*.

When granule extracts prepared in the presence of protease inhibitors were compared by SDS-PAGE with the acetate-EDTA extract, they appeared to contain two to four protein bands in the 30,000- to 50,000-molecular-weight range that were not present in the acetate-EDTA extract. This indicates that the inhibitors exert some effect on the granule proteases. However, Western blot analyses of the two types of extracts did not show any major change in the amount of peptides that reacted with BP97.

TABLE 2. Relationship between hydrophobicity and bactericidal activity in a family of granule polypeptides reacting with MAb BP97 and showing identical migration on SDS-PAGE[a]

Acetonitrile (%)	Mol wt (10^3)	Immunoreactivity	MIC (μg/ml)	
			S. aureus	*E. coli*
26.0 ± 0.1	4.3	++	>100	100
27.5	4.3	++++	>100	50
29.7 ± 0.1	4.3	+	>100	100
30.5 ± 0.1	4.3	+++	>100	25
31.9 ± 0.2	4.3	+++	>100	>100
32.6 ± 0.2	4.3	++++	>100	>100
34.7 ± 0.1	4.3	+++	>100	>100

[a] Experimental details are as described in footnote *a* of Table 1.

DISCUSSION

A variety of cationic polypeptides purified from the granules of rabbit, human, and bovine neutrophils have been reported to exert antimicrobial or antiviral activity in vitro (3, 5, 6, 8, 18, 19, 21, 25, 26). They differ in size, spectrum of activity, and efficiency.

Here we reported that several polypeptides of different sizes purified from the granules of bovine neutrophils are specifically recognized by a MAb raised against a semipure bactericidal protein fraction that was previously isolated from the same source (18). Of these peptides, those that fell in the molecular weight range of 4,000 to 8,000 and that possessed the greatest positive charge inhibited the growth of *E. coli* in a physiological medium at MICs of 12 to 50 μg/ml. In addition, a 1,600-molecular-weight peptide was also active against *S. aureus* and *S. epidermidis*, with MICs of 3 to 8 μg/ml. This peptide had the same molecular weight, reactivity to MAb BP97, and selectivity of antibacterial action of a dodecapeptide that we have purified from granules of bovine neutrophils by conventional chromatography and shown to contain four Arg residues interspersed among hydrophobic amino acid residues (D. Romeo, B. Skerlavaj, M. Bologness, and R. Gennaro, J. Biol. Chem., in press).

In general, among the defensins of both rabbit and human neutrophils (19, 20), as in the case described here, the most cationic peptides are the most active as antibacterial agents. For instance, the human defensin human neutrophil peptide 3 (HNP-3), which has an additional negatively charged amino acid residue (Asp) at the N terminus, is less active than HNP-1 and HNP-2 against a variety of microorganisms. The apparently constant correlation between positive charge and antibacterial effects may likely be ascribed to an initial attack of the antibacterial peptides to the bacterial membrane(s) by charge-charge interactions, followed by secondary hydrophobic interactions (25).

The immunoreactivity of the antimicrobial peptides to MAb BP97 is shared by larger, less hydrophilic polypeptides that are also present in the granule extracts. MAb BP97 appears to be specific for granule proteins, because it is able to discriminate among several cationic peptides, including a protamine-like polypeptide from *Ensis minor* (9), which has an Arg residue content very similar to those of the 4,300- and 7,300-molecular-weight peptides (unpublished data).

One possible explanation of our findings would be that the antimicrobial peptides of bovine neutrophils arise from the processing of larger progenitor molecules. In several eucaryotic systems, secretory products, including the antibacterial proteins attacins that are present in the hemolymph of the pupae of the silk moth *Hyalophora cecropia*, have actually been shown to be proteolytically cleaved from larger precursor molecules (4, 11, 14). In addition, to generate multiple products with separate functions, this process can provide

an efficient and economic system for synthesizing several copies of the same peptide within a single precursor molecule.

The antimicrobial defensins of human neutrophils HNP-1, HNP-2, and HNP-3 are also likely derived from a common precursor. In fact, they contain an identical sequence of 29 amino acids, with an additional amino acid residue at the N terminus of HNP-1 and HNP-3 (Ala and Asp, respectively) (20). Furthermore, it is interesting that some antibiotic peptides produced by bacteria by way of the protein synthetic mechanism also appear to be derived from a progenitor protein. In fact, it has been shown that cationic proteins of *Streptococcus lactis* or of a particular strain of *Bacillus subtilis* are converted in vitro by crude extracts of the producing organism into the 34-amino-acid antibiotics nisin and subtilin, respectively (16).

In preliminary experiments, we searched for additional support to our hypothesis by supplementing the granule extraction medium with inhibitors of acid proteases that are putatively involved in the processing of a large precursor into the 1,600- to 8,000-molecular-weight antimicrobial peptides. The presence of these inhibitors did not significantly change the yield of BP97-reactive peptides in the extract, as seen by Western blot analyses. These results are consistent with the observation by Spitznagel and co-workers (21) that treatment of human neutrophils with the serine protease inhibitor diisopropylisofluorophosphate causes only minor differences in the SDS-PAGE protein pattern of acid extracts of granules, without changing the bactericidal 50% lethal dose of the extracts.

The proteolytic cleavage of the putative precursor(s) may thus occur before storage of the resulting products into the granules or may be carried out in the granule extracts by a protease whose activity is not blocked by the inhibitors that we used. We are exploring the possibility of approaching this problem by identifying and characterizing the mRNA(s) for the putative precursor(s) in undifferentiated leukocytes.

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